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Glycosylated Hemoglobin Assay and Oral Glucose Tolerance Test Compared for Detection of Diabetes Mellitus

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We compared the oral glucose tolerance test (I) as evaluated by six commonly used scoring methods and total glycohemoglobin assay (II) with respect to their value in the diagnosis of diabetes mellitus. Depending on the evaluation method used for I, 16.7 to 64.3% of those subjects diagnosed as diabetic or borderline by this test were judged to be normal by II. The best agreement was between II and the Unger evaluation method. High-density-lipoprotein cholesterol, which showed an inverse correlation with II, was decreased in subjects judged to be diabetic by the Unger method. We conclude that the utilization of II measurement as a screening method for diabetes mellitus is consistent with a conservative approach to the diagnosis of diabetes.

Additional Keyphrases: *high-density-lipoprotein cholesterol* · *diagnostic aids* · *intermethod comparison*

For many years, diagnosis of diabetes mellitus has depended primarily on results of oral glucose-loading tests. Fasting hyperglycemia, a late manifestation of diabetes, is not generally useful for evaluating the presence or severity of glucose intolerance. The oral glucose tolerance test (oGTT)[1] has been criticized as leading to overdiagnosis of diabetes (*1*, *2*) because of the scoring systems used to evaluate test results. Furthermore, the results of this test can vary with such factors as age, presence of infection, previous diet, time of testing, and physical activity of the subject.

The glycosylated hemoglobin components (HbA_1)[2] are increased in diabetics (*3*). This assay has found acceptance as a means of measuring the degree of long-term regulation of blood glucose in known diabetics, but the potential value of the HbA_1 assay as a screening technique for diabetes has not as yet been thoroughly evaluated. Koenig et al. (*4*) reported a good correlation between HbA_{1c} and the area under the oGTT curve. Santiago et al. (*5*) reported a correlation between HbA_{1c} and the response to glucose loading at 1 and 2 h of the oGTT.

We have evaluated the oGTT by six scoring methods: Wilkerson (W) (*6*), Fajans-Conn (FC) (*6*), The University Group Diabetes Program (UGDP) (*6*), Japan Diabetic Society (JDS) (*7*), U.S. Public Health Service (USPHS) (*2*), and Unger (U) (*8*). We compared the results with each other and with results of the HbA_1 assay.

Since high-density-lipoprotein cholesterol (HDL-cholesterol) is decreased in diabetics (*9*), we also explored its relationship to HbA_1 and its potential usefulness in identifying diabetics.

Materials and Methods

Subjects

A total of 35 apparently healthy subjects, 23 women and 12 men ranging in age from 23 to 80 years, served as a control group. A total of 27 patients (15 women, 12 men), hospitalized for uncontrolled diabetes mellitus and ranging in age from 23 to 82 years old, were studied. All were on insulin therapy. The mean serum glucose (fasting) was 1.95 g/L (range 1.20–4.30 g/L).

We evaluated results of 44 oGTT's. Subjects in the glucose load study ranged in age from 20 to 70 years and consisted of 22 women and 22 men. The oGTT was administered by giving a 100-g load of glucose (Dextol; Sherwood, Inc., St. Louis, MO 63103) after a 12-h (overnight) fast. Samples for HbA_1 and HDL-cholesterol measurement were obtained, along with a sample for glucose assay, immediately before Dextol administration.

Procedures

HbA_1 was determined by mini-column chromatography based on the separation method of Trivelli et al. (*10*). Minicolumns, 0.8 × 4 cm, packed with Bio-Rex 70 cation-exchange resin (Bio-Rad Laboratories, Richmond, CA 94804) were used to separate total HbA_1 from unglycosylated hemoglobin. The resin had been washed with dilute HCl and water, then equilibrated with the eluting buffer, fines being removed at the buffer-equilibration step. Columns were poured as a slurry in buffer and allowed to settle overnight at room temperature. The elution buffer (pH 6.70 ± 0.05) contained, per liter, 4.59 g of sodium dihydrogen phosphate monohydrate, 1.18 g of anhydrous disodium hydrogen phosphate, 650 mg of potassium cyanide, and 650 mg of sodium azide. Blood samples with disodium ethylenediaminetetraacetate as anticoagulant were hemolyzed by mixing 100 μL of whole blood with 400 μL of a 5 mL/L solution of Triton X-100 surfactant (Sigma Chemical

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[1] The following nonstandard abbreviations are used in this paper: oGTT, oral glucose tolerance test; HDL-cholesterol, high-density-lipoprotein cholesterol; HbA_1, total glycosylated hemoglobin; HbA_{1c}, glycosylated hemoglobin fraction c. In addition, for brevity, the oral glucose tolerance test as scored by a specific evaluation method is designated as oGTT followed by the test abbreviation W, FC, UGDP, JDS, USPHS, or U (see text).

[2] HbA_1 is composed of at least four fractions, $HbA_{1a+b+c+d}$. The HbA_{1c} fraction is the most increased in diabetic patients. However, there are significant increases in the other fractions. We measured total glycohemoglobin in this study because the small increases in Hb_{1a+b+d}, when added to the increase in HbA_{1c}, tend to enhance the distinction between diabetics and nondiabetics.

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Co., St. Louis, MO 63178) and allowing the mixture to stand for 10 min at room temperature, with occasional agitation. Fifty microliters of each hemolysate was gently added to the top of each column, followed by about 250 µL of buffer. A standard obtained from Isolab, Inc., Akron, OH 44321 (Hemoglobin Mini-Column Standard, QS-79) was included in each run. Four milliliters of buffer was added to each column and the eluates were collected.

While the columns were developing, total hemoglobin was determined by adding 10 µL of each hemolysate and the standard to 10.00 mL of buffer. The absorbance (*A*) of each sample and standard, both that of the eluate and that for total hemoglobin for each, was measured at 415 nm in a 1.00-cm cuvet referenced to water. The labeled value for the standard was obtained by the supplier, using the Trivelli macrocolumn chromatographic method (*10*). We found the column-chromatographic method to be sensitive to ambient temperature and column packing. We corrected for this by multiplying the HbA_1 value obtained from equation 1 for each patient's sample by a factor derived from the ratio of the labeled value for the standard to the value determined for the standard in the run (equation 2).

$$\%HbA_1 = (A \text{ of eluate/total } A \text{ placed on column}) \times 100 \quad (1)$$

where "total *A* placed on column" = (50 µL/10 µL) × (10 mL/4 mL) × *A*, for total hemoglobin sample. This simplifies to 12.5 × *A* for total hemoglobin sample.

$$\%HbA_1(\text{corrected}) = \%HbA_1 \times (\text{labeled value for std./value determined for std.}) \quad (2)$$

Corrected HbA_1 values are used in our study.

The coefficients of variation intra-run were 3.0% (for HbA_1 values of 5 to 10%) and 4.0% (for HbA_1 values exceeding 10%). The corresponding inter-run coefficients of variation were 4.0 and 1.0%.

HDL-cholesterol was quantitated by polyacrylamide gel electrophoresis as described by Muñiz (*11*), except that pooled sera of known HDL-cholesterol content served as standards; the HDL-cholesterol concentration of each standard had been determined by phosphotungstate–magnesium precipitation followed by enzymic assay of cholesterol in the supernate (*12*).

We used a standard curve relating HDL-cholesterol concentration to the area under the HDL peak of the densitometric scan. HDL-cholesterol as determined by this method correlated satisfactorily (r = 0.96, Figure 1) with the phosphotungstate–magnesium precipitation method.

Venous blood was collected with disodium ethylenediaminetetraacetate (1 g/L) as anticoagulant and stored at 4 °C for up to three days for HbA_1 analysis. A portion of each whole-blood sample was centrifuged, and the plasma was removed and stored at 4 °C for total cholesterol and HDL-cholesterol assay.

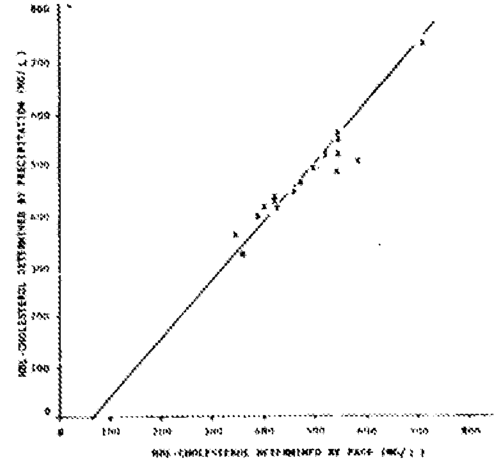

Fig. 1. Correlation between concentration of HDL-cholesterol as determined by polyacrylamide gel electrophoresis, with use of standards of known value, and that determined by phosphotungstate–$MgCl_2$ precipitation
n = 17, r = 0.96, $y = 1.08x - 32.6$

Evaluation of the Oral Glucose Tolerance Test

The oGTT for the detection of diabetes mellitus was evaluated by the following methods:

(*a*) *The Wilkerson Point System (oGTT-W) (6):*

Time, h	g/L	Points
0	>1.29	1
1	>1.94	½
2	>1.39	½
3	>1.29	1

A score of 2 points or more indicates diabetes. A score of 1 or 1½ is considered borderline.

(*b*) *The Fajans-Conn Criteria (oGTT-FC) (6):*

Time, h	g/L
1	>1.84
1½	>1.64
2	>1.39

If all criteria are exceeded the individual is considered diabetic. In our study, a subject who exceeded one or two of the above criteria was considered borderline.

(*c*) *The University Group Diabetic Program (oGTT-UGDP) (6):*

An individual is considered to be diabetic if the sum of the fasting, 1-, 2-, and 3-h glucose values exceeds 5.99 g/L. No criterion for borderline subjects was established.

(*d*) *Japan Diabetic Society (oGTT-JDS) (7):*

Time, h	g/L
1	>1.80
2	>1.60

When both of the above criteria are exceeded the individual is considered diabetic. The subject is considered borderline if one of the criteria is exceeded, or if at 1 and 2 h the glucose concentrations are 1.60 to 1.80 and 1.20 to 1.60 g/L, respectively.

(*e*) *U.S. Public Health Service (oGTT-USPHS) (2):*

Time, h	g/L	Points
0	>1.24	1
1	>1.94	½
2	>1.39	½
3	>1.24	1

A score of 2 points or more indicates diabetes; 1 or 1½ points is considered borderline.

(*f*) *Unger Evaluation Method (oGTT-U) (8):*

Time, h	g/L
1	>2.59
2	>2.19

When both criteria are exceeded the subject is considered diabetic. If one of the criteria is exceeded the subject is considered borderline.

Evaluation of the Total Glycohemoglobin (HbA_1) Assay

A value greater than 9.5% was considered indicative of di-

Table 1. HbA_1 and HDL-Cholesterol Concentrations in Healthy and Diabetic Subjects

Sex	n	Age: Mean	Age: Median	HbA_1 (%)	HDL-cholesterol (mg/L)
			Healthy—our study		
Men	12	47.8 (12.4)	40	7.20 (0.76)	478 (96)
Women	23	42.3 (17.9)	49	7.41 (1.08)	529 (117)
			Framingham study		
Men	42	3 (60–69)[a]			455 (134)
Women	57	5 (60–69)[a]			568 (154)
			Diabetic subjects—our study		
Men	12	68.1 (13.3)	67	12.2 (2.1)[b]	337 (127)[b,c]
Women	15	61.7 (14.4)	63	12.3 (3.1)[d]	411 (112)[d,e]

[a] Age range.
[b] Significantly different from healthy men at $p < 0.001$.
[c] Significantly different from Framingham men at $p < 0.01$.
[d] Significantly different from healthy women at $p < 0.001$.
[e] Significantly different from Framingham women at $p < 0.01$.
Standard deviations are in parentheses.

Table 2. Comparison of Two Oral Glucose Tolerance Test Scoring Methods

Group	n	Sex	Mean age, years	HbA_1, %	HDL-cholesterol, mg/L	Cholesterol, g/L
1	8	♂	38.3 (17.3)	7.44 (0.90)	560 (145)	2.07 (0.55)
	8	♀				
2	3	♂	66.0 (6.84)	7.62 (0.49)	498 (161)	2.16 (0.46)
	3	♀				
3	4	♂	55.8 (17.7)	10.2 (1.50)[a]	350 (114)[a]	2.21 (0.25)
	4	♀				

[a] Significantly different from Group 1 at $p < 0.01$.
Group 1—normal by FC standards; normal by U criteria.
Group 2—abnormal by FC standards; normal by U criteria.
Group 3—abnormal by FC standards; abnormal by U criteria.
Standard deviations are in parentheses.

abetes for the purposes of our study. Values of 9.1 to 9.4% are considered borderline in our study. These criteria are based on the normal range that was found in our study for a population of 35 healthy subjects (Table 1).

Evaluation of the HDL-Cholesterol Assay

Men with HDL-cholesterol values of <400 mg/L and women with values <500 mg/L were considered to have a greater risk of being diabetic. The data in Table 1 demonstrating means of 337 mg/L for diabetic men and 411 mg/L for diabetic women served as guidelines for the establishment of these limits.

Results

Glycohemoglobin (HbA_1)

The correlation between values for HbA_1 and serum glucose during fasting was examined by linear regression analysis in a population of healthy and diabetic subjects (Figure 2). HbA_1 was significantly correlated ($r = 0.74$, $p < 0.001$) with serum glucose in this population of 63 individuals. There was no significant correlation ($r = 0.07$) when healthy subjects were analyzed separately but there was a significant correlation ($r = 0.57$, $p < 0.001$) in the diabetic population. There was a highly significant HbA_1 increase in hospitalized uncontrolled diabetics (Table 1). The above results are consistent with previously published research (*13*).

We found a significant increase in HbA_1 in a group of individuals judged to be diabetic by both the oGTT-FC and oGTT-U (Group 3, Table 2). There was no increase in HbA_1 in a group of subjects considered abnormal by oGTT-FC criteria but normal by oGTT-U (Group 2). Table 3 shows additional evidence that HbA_1 agrees with the oGTT only when the oGTT is evaluated by conservative standards. Forty-four casually chosen oGTT's were scored by the eight evaluation methods described in the *Materials and Methods* section. Table 3 shows the frequency of agreement between paired scoring systems. HbA_1 has a high frequency of agreement with oGTT-U (86.4%) and oGTT-UGDP (84.1%). When we focus

Table 3. Frequency (in Per Cent) of Agreement between Various Tolerance Test Scoring Methods, HbA_1, and HDL-Cholesterol

	FC	UGPD	JDS	USPHS	U	HbA_1	HDL-cholesterol
W	84.1	95.5	86.4	93.2	81.8	77.3	76.2
	FC	81.8	84.1	79.5	77.3	65.9	71.4
		UGDP	79.5	90.9	90.9	84.1	71.4
			JDS	79.5	75.0	65.9	66.7
				USPHS	84.1	75.0	81.0
					U	86.4	76.2
						HbA_1	76.2

Each subject was designated normal, diabetic, or borderline by each scoring method. The above table presents the per cent agreement between two evaluation methods. All comparisons involved 44 subjects, except HDL-cholesterol comparisons, which involved 21 subjects.

Table 4. Comparison of the Diagnosis of Diabetes by HbA_1 and oGTT

	W	FC	UGDP	JDS	U	USPHS
No. subjects judged diabetic or borderline by oGTT evaluation method	11	13	8	14	6	9
No. of above subjects scored normal by HbA_1	5	8	3	9	1	4
Per cent that would be considered normal by HbA_1	45.5	61.5	37.5	64.3	16.7	44.4

only on those subjects diagnosed as diabetic or borderline by the six most commonly used oGTT scoring systems, we find that HbA_1 classifies 16.7 to 64.3% of them as normal (Table 4).

HDL-Cholesterol

In determining HDL-cholesterol we used standards in each electrophoresis run. As a result, HDL-cholesterol showed a good correlation with the phosphotungstate–magnesium precipitation method ($r = 0.96$, Figure 1). There was an inverse relationship between HDL-cholesterol and HbA_1 ($r = -0.42$, $p < 0.001$) in a combined healthy and diabetic population (Figure 3). Table 1 shows that concomitant with an increase in HbA_1 there is a significant decrease in HDL-cholesterol in diabetics. Although a relatively small healthy population was used, our values for mean HDL-cholesterol corresponded closely to data from the Framingham Study (*14* and footnote[3]). Furthermore, HDL-cholesterol concentrations were significantly less in both men and women in our diabetic population than in the corresponding normal population in the Framingham Study. Table 2 shows that individuals classified normal by oGTT-U and diabetic by oGTT-FC have normal HDL-cholesterol values. In contrast, our population of individuals diagnosed as diabetic by both scoring methods has significantly subnormal HDL-cholesterol values and increased HbA_1 values.

Subnormal HDL-cholesterol corresponded with a frequency of 66.7 to 86.4% when paired with the commonly used oGTT scoring methods (W, FC, UGDP, JDS, USPHS, U). In comparison, there was agreement with HbA_1 in 16 of 21 subjects (76.2%, Table 3).

Discussion

Our data indicate that 37 to 64% of those individuals judged to be diabetic by the oGTT, as evaluated by the commonly used scoring methods, would be classified as non-diabetic by results of the HbA_1 assay. When all 44 pairs were compared, the frequency with which there was diagnostic agreement between HbA_1 and the oGTT ranged from 65.9 to 86.4%. Only two evaluation procedures showed >80% agreement with HbA_1: oGTT-U (86.4%) and oGTT-UGDP (84.1%). oGTT-U is the most conservative approach thus far reported for evaluating the oGTT. oGTT-UGDP is directly proportional to the area under the oGTT curve. Koenig et al. (*4*) reported a correlation between the area under the oGTT curve and HbA_{1c}. Our analysis of values for HDL-cholesterol in diabetics also suggests a conservative approach to the diagnosis of diabetes mellitus. HDL-cholesterol reportedly (*9*) correlates inversely with serum glucose in diabetics. We found a significant inverse correlation between HDL-cholesterol and HbA_1. HDL-cholesterol values were significantly smaller in subjects evaluated as diabetics by both oGTT-FC and oGTT-U. This finding coincided with a significant HbA_1 increase. We found no significant differences from normal for either HDL-cholesterol or HbA_1 in subjects scored as diabetic by oGTT-FC but non-diabetic by oGTT-U. Because diabetes mellitus is considered to be a risk factor for coronary disease, it is not surprising that diabetics have smaller HDL-cholesterol concentrations.

HbA_1 assay as a diabetes-screening technique has the advantage of being insensitive to all those factors that affect the results of the oGTT. The only interferences reported thus far are shortened erythrocyte survival time and the presence of fetal hemoglobin. Major drawbacks to acceptance of the HbA_1 assay have been the lack of valid controls for evaluating the test and time-consuming methods. Recent advances in "high-performance" liquid chromatography (*15*) and the development of a semi-automated macrocolumn method (*16*) partly solve the problem. The mini-column chromatographic assay for HbA_1 that we have described is simple, economical,

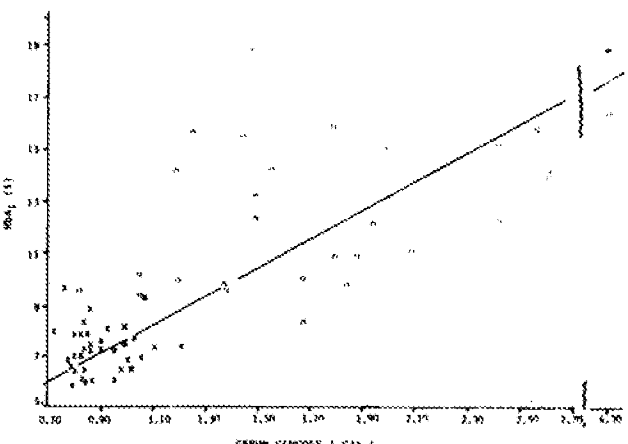

Fig. 2. Correlation between HbA_1 and serum glucose in healthy (x) and diabetic (o) subjects
$n = 73$, $r = 0.744$, $p < 0.001$, $y = 3.06x + 5.52$

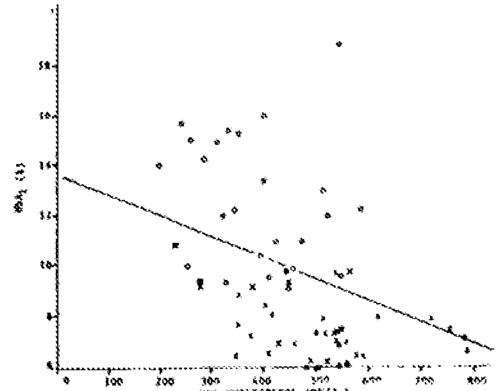

Fig. 3. Correlation between HDL-cholesterol and HbA_1 in healthy (x) and diabetic (o) subjects
$n = 63$, $r = -0.419$, $p < 0.001$, $y = -0.0091x + 13.9$

[3] Standard deviations from the Framingham Study were kindly provided by M. C. Hjortland, National Heart, Lung and Blood Institute, Bethesda, MD 20014.

reproducible, and feasible for use in a small hospital laboratory. The utilization of a commercially available standard allows for corrections of inter-run differences caused by the sensitivity of the column technique to ambient temperature and column packing. Although not feasible for fast determination of HbA_1 in large numbers of samples, high-performance liquid chromatography could be used to accurately quantitate the HbA_1 content of the standards used to calibrate the column method.

We did not attempt to relate HbA_1 or any of the oGTT evaluation methods to the microangiopathic sequelae of diabetes. It is surprising and disappointing that HbA_{1c} fails to correlate with basement membrane thickening (*4, 17*).

Interestingly, Peterson et al. reported (*18*) a correlation between serum triglyceride and HbA_{1c} in diabetes mellitus, but we found no correlation between HbA_1 and serum cholesterol.

This study clearly demonstrates that the use of HbA_1 as a detection assay for diabetes mellitus necessitates the acceptance of conservative criteria for the diagnosis of diabetes.

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